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PRINCIPAL INVESTIGATOR: Richard H. C. San, Ph.D.

CONTRACTING ORGANIZATION: Microbiological Associates, Inc.
Rockville, Maryland 20850

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Rockville, Maryland 20850

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The purpose of this study is to evaluate the use of cells from a mouse macrophage cell line RAW 264.7 as an *in vitro* test for determining the cytotoxicity and mutagenicity of particulates potentially hazardous to the respiratory system. Mutagenicity assays were conducted on the hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene locus. The mutant frequency in the untreated control in some experiments was substantially higher than the spontaneous frequency of between 0 and 10 mutants per 10^6 cells reported by Hitchins *et al.* (1992). It remains to be determined as to whether the variability in spontaneous mutant frequency is an inherent feature of this cell line. The inability to demonstrate mutagenicity with low fire or high fire beryllium oxide indicates that the assay procedure still requires further refinement. No mutagenic activity was demonstrated with red dye mix. A dose-related increase in mutant frequency was observed with 10% trinitrotoluene (TNT)-contaminated compost in only one experiment. Since the TNT-contaminated compost was not tested at concentrations higher than 100 µg/mL using the 18-hour extended treatment regimen, additional experiments are warranted prior to making comments on the potential mutagenicity of soils contaminated with munitions.

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Richard X October 16, 1997

PI - Signature Date

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INTRODUCTION

Toxic assault through the respiratory tract is a major threat in both working and domestic environments. Most of these toxic materials are small, soluble molecules which can be easily tested for cytotoxicity and genotoxicity by current short term tests. However, around 10% of airborne pollution is made up of particulates (Amdur, 1986). These may be smokes, fly ash, mineral dusts or other particles of 5 microns or less and therefore able to pass into the lungs (Menzel and Amdur, 1986). Since there is no direct assay for particulates, extracts of particulate materials are often tested in the established assays (Brusick, 1982; Zelikoff, 1985). This approach assumes that the active portion of the particulate is extractable and that the non-extractable solid portion of the particulate is inactive. However, there is evidence that certain solids are directly genotoxic (asbestos, glass fibers and certain metals) and that the form and size of the particle is critical in determining its toxicity (Sincook *et al.*, 1982; Hesterberg *et al.*, 1983). These properties would be missed in an assay based on extraction of particulate material. Thus, there is a need for an *in vitro* assay system utilizing cells which can actively phagocytize the particles to be tested and allowing assessment of their effects through several endpoints.

Attempts to use traditional short term tests to assess cytotoxicity and genotoxicity of particulates directly have met with varying success. Zelikoff *et al.* (1985) used V79 Chinese hamster lung fibroblasts to study the cytotoxicity of silica, alumina, wood charcoal and coal fly ash. They were able to demonstrate phagocytosis of these particles but it is not clear what percentage of the particles were absorbed. Costa and Heck (1982) studied the ability of Chinese hamster ovary cells to phagocytize specific crystalline and amorphous nickel, cobalt, copper and cadmium coupounds. Between 14% and 49% of the cells ingested the crystalline salts while only 3% to 6% of the cells phagocytized the amorphous salts. These results were consistent with results of the Syrian hamster embryo transformation assay which showed greater transformation by the crystalline salts. The percentage of cells which actually phagocytized the particles was low. Rossman *et al.* (1987) reviewed the genetic toxicity of metal compounds in *in vitro* systems. They found that bacterial systems give mixed results in comparison to *in vivo* systems. Genotoxic effects of soluble salts were detected in these systems, while the effects of insoluble salts were not. Cultured, primary rat liver cells were a better model for determining cytotoxicity than bacteria. However, these cells were nondividing and needed to be obtained directly from donor animals and could not be easily used in mutagenicity assays.

Macrophages play an important role in the lung in trapping, concentrating, and processing, through both superoxide and lysosomal enzyme systems, potentially genotoxic particles (Drath *et al.*, 1976). Macrophages ingest most of the particles which pass into the bronchiolar and alveolar regions of the lungs. Thus, macrophages would be ideal target cells in an assay for assessment of cytotoxicity of particulates. Freshly isolated alverlar macrophages have been used as targets for this purpose. Metals (Castranova *et al.*, 1980), fly ash (Hill *et al.*, 1982), tars and other combustion products (Hill *et al.*, 1983), and volcanic dust (McLemore *et al.*, 1984) have been studied with a number of endpoints. However, as these are primary, nondividing cells, they cannot be used for study of many genotoxic endpoints. Mutation assays require cell division to allow fixation and expression of genotoxic lesions. Therefore, a continuous, dividing cell line, with the phagocytic and metabolic properties of a normal macrophage, would combine the desired characteristics for an *in vitro* assay to measure the genotoxic potential of particulates.

The purpose of the study presented in this report is the development and validation of an inexpensive and rapid *in vitro* test for determining the cytotoxicity and genotoxicity of particulates potentially hazardous to the respiratory system. The short term tests currently available were designed for the testing of soluble test materials and were adapted for the study of particulates with target cells possessing limited phagocytic ability. The assay system evaluated in this report used a mouse macrophage cell line, RAW 264.7, which is avidly phagocytic and will undergo a respiratory burst. This cell line grows readily in culture, a feature which will allow both cytotoxic and genotoxic endpoints to be monitored. Preliminary studies have been conducted to establish the basic parameters for both a cytotoxicity assay and an HGPRT gene based forward mutation assay. These studies showed that RAW 264.7 cells exhibited a dose dependent but differential cytotoxic response to high versus low fire beryllium oxide particles and dose dependent mutagenic responses to two soluble mutagens, ultraviolet radiation (UVC) and ethyl methanesulfonate. The goals for the present study are to complete the work on establishing optimal conditions for the assays, validate the cytotoxicity assay with known toxic particulates and the mutation assay with several known classes of mutagenic particulates.

MATERIALS AND METHODS

Test Materials

Low Fire Beryllium Oxide and High Fire Beryllium Oxide were obtained as a gift from Dr. John H. Harbell, Institute for *In Vitro* Sciences, Gaithersburg, Maryland. The following test materials were provided by the project officer: trinitrotoluene (TNT)-contaminated compost, 10% TNT-contaminated compost, uncontaminated soil sample, and red dye mix (a mixture of Solvent Red 1 and Disperse Red 11).

Cell Line and Culture Conditions

The RAW 264.7 macrophage cell line was derived from an Abelson leukemia virus infected BAB/14 mouse by Raschke and co-workers (1978). The cell line is avidly phagocytic towards both antibody coated and uncoated particles, has Fc and complement receptors, and produces lysozyme. These cells have been shown to actively phagocytose and kill bacteria, as well as undergo the "respiratory burst" and limited release of superoxide characteristic of alveolar macrophages (McGown *et al.*, 1985a). The symosan induced respiratory burst was measured after poisoning mitochondrial oxygen consumption with KCN and was found to consume between 2 and 3 nmoles per 10^6 cells per minute. Thus, this cell line combines the avid phagocytic activity and important metabolic properties of the normal macrophage with the ability to grow and divide in culture. The use of this functional macrophage cell line allows the study of genetic endpoints, as well as cytotoxicity, without the need to sacrifice animal donors for each assay. In addition, a number of physiologically relevant studies will also be possible with this assay system. The study of combined soluble mutagen and particulate, as might occur with smokes, would be greatly facilitated by this target cell which is sensitive to both. Similarly, the lung cells may be subject to the combined effects of the respiratory burst and toxic challenge and this system would allow the study of such a synergy.

Several pertinent preliminary studies on the RAW 264.7 cells and culture system have been completed. These include media and serum requirements, growth in suspension culture,

phagocytic activity, population doubling time, clonability, propensity to form colonies on plastic, and chromosome number.

The RAW 264.7 cell line was originally isolated in minimal essential medium (MEM) with 10% fetal bovine serum. It grows well in RPMI 1640 and Ham's F12 media without hypoxanthine. The cells attach to wettable surfaces (polystyrene or glass) but not to polypropylene or siliconized glass. Thus, they can be cultured either in attached or suspension culture. Phagocytic activity is greater and more uniform in suspension culture. After a 2-hour challenge, over 95% of the cells were observed to have ingested carbon or 2-micron latex particles (J. Harbell, personal communication). The population doubling time, in suspension, is between 18 and 20 hours, and the population continues to divide after ingesting particles. The cells can be cloned and they form discrete colonies on plastic, two characteristics essential to a clonal mutation assay system. The cell line is pseudodiploid (modal count of 40). An $HGPRT^{-/-}$ subclone was produced for use in reconstruction experiments. Transmission electron micrographs of cell clusters grown in suspension or attached culture did not show gap junctions. Therefore, cross feeding between mutants and nonmutants during the 6-thioguanine mutant selection phase of the HGPRT assay would seem unlikely. For, example, cross feeding between mutant and nonmutant CHO cells limits the cell density to only $2x10^5$ cells on each mutant selection plate.

The standard culture medium used in this study was RPMI 1640 supplemented with 10% fetal bovine serum. This culture medium was used for both the cytotoxicity and mutagenicity assay, and for both attached and suspension cultures. Unless specified otherwise, cells were exposed to the test materials in suspension culture. To remove uningested test material, if appropriate, the cells were allowed to attach to flasks and the monolayer rinsed to remove the free particles. Cells were removed from the plastic, for counting or passaging, by treatment with Ca^{++} and Mg^{++} free Hanks' balanced salt solution (HBSS). Ethylenediaminetetraacetic acid (EDTA; 0.02%) might also be added to facilitate cell release. The use of trypsin was avoided as it tended to activate the membrane of the RAW 264.7 cells.

Cytotoxicity Assay

Cytotoxicity can be manifested in many forms including loss of differentiated function, cessation of cell replication, and/or cell death. The RAW 264.7 cell line is appropriate for the study of all of these endpoints. McGown *et al.* (1985b) have shown that trivalent arsenicals can inhibit respiratory burst and bacterial cell killing (differentiated function), reduce cell replication, and kill cells outright depending on the dose. Preliminary studies demonstrated that changes in population number determined by cloning cells can be used for measuring cytotoxicity. Toxicity could also be determined by assessment of active uptake of particulates. The loss of differentiated function can be determined through measurement of changes in the respiratory burst induced by symosan particles.

For the cytotoxicity assay, 10 ml suspension cultures at $5x10^4$ cells per ml were prepared in 15 ml polypropylene tubes. Serial dilutions of the test material were added to the appropriately labelled tubes. Untreated and carrier/solvent (if appropriate) control cultures were also prepared. The cells were exposed, in suspension culture on a roller drum, for 5 hours (unless specified otherwise) and then counted. For assessment of toxicity based on determination of uptake of particulates, an aliquot of the cells was withdrawn, and the cells were fixed with methanol,

stained with 10% Giemsa and the number of cells which ingested particles determined by examination under a microscope. The remainder of the cells was carried forward to determine cloning efficiency. In cases where separation of the uningested test material from the cells was desired, the cells would be plated into 25 cm^2 flasks and allowed to attach for 30 minutes. The monolayer was then rinsed with culture medium to remove the uningested test material. The cultures were fed with fresh medium and allowed to grow for 18 to 24 hours. Cells were then detached, counted and reseeded at approximately 3 cells per well in 96-well microtiter plates or at 100 cells per 60 mm petri plate. After 8 to 10 days, the colonies were fixed with methanol, stained with 10% Giemsa, counted and evaluated for cytotoxic effects. Data were presented as the relative cloning efficiency of the treated populations as compared to that in the untreated control population.

Mutagenicity Assay

The hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene locus on the X chromosome has been used as a target for forward mutation studies because mammalian cells are naturally hemizygous at this locus. The RAW 264.7 cell line is hemizygous with a spontaneous frequency of between 0 and 10 mutants per 10*6* cells (Hitchins *et al.*, 1992). The cells will form colonies, do not appear to form gap junctions, and are not inhibited by rat liver S9 activation. These characteristics are necessary characteristics for a target cell line in a forward mutation assay.

In preliminary experiments, the $HGPRT^{-/-}$ subclone was developed and used in reconstruction experiments to determine parameters for the assay using the same approach as that used by Hsie *et al.* (1974) in the development of the HGPRT assay in CHO cells. These experiments allowed the determination of the optimal mutant expression period required to achieve maximum mutant frequency yields, the mutant selection period, the amount of selective agent (6-thioguanine) required, and the maximum cell plating density during the mutant selection phase. Two mutagens, ultraviolet radiation (UVC; Hitchins *et al.*, 1992) and ethyl methanesulfonate (EMS; Bigger, unpublished data), were tested in the assay and dose dependent increases comparable to those achieved in the CHO/HGPRT assay were achieved in the RAW 264.7 cell assay.

For the mutagenicity assay, cells were treated with test article and a portion of the treated cells were used to determine cytotoxicity as described above for the cytotoxicity assay. For mutagenic evaluation, the remainder of the cells were returned to culture for five to seven days to allow expression of the mutation at the HGPRT locus. At the time of selection, cells were seeded at 2×10^5 cells per 60 mm petri dish (or, at 5×10^3 cells/well in a 96-well microtiter plate) in the presence of 4×10^{-5}M 6-thioguanine; for cloning efficiency, cells were seeded at 100 cells per 60 mm petri dish (or, at 3 cells/well in a 96-well microtiter plate).

For seeding in 60 mm petri dishes, the computation of cloning efficiency and mutant frequency was as follows:

$$\text{Cloning efficiency (\%)} = \frac{\text{Total colonies counted}}{\text{Dishes counted} \times 100 \text{ cells}} \times 100$$

$$\text{Mutants/}10^6\text{ clonable cells} = \frac{\text{Total mutant colonies} \times 10^6}{\text{Number selection dishes} \times \text{cloning efficiency} \times 2 \times 10^5 \text{ cells}}$$

For seeding in 96-well microtiter plates, the computation of cloning efficiency and mutant frequency was as follows:

$$\text{Cloning efficiency} = \frac{\text{-Natural log (number of empty wells/total number of wells)}}{\text{Number of cells seeded per well}} \times 100$$

$$\text{Mutants/}10^6\text{ clonable cells} = \frac{\text{Cloning efficiency on selection plates}}{\text{Cloning efficiency on non-selection plates}}$$

RESULTS

Low and High Fire Berillium Oxide (BeO). Preliminary studies were conducted with low and high fire BeO particles (Bigger, unpublished data). RAW 264.7 cells and Chinese hamster ovary (CHO) cells were compared with respect to their susceptibility to the toxic effects of these particles. Cells from both of these lines showed a dose dependent cytotoxic response to the low fire particles but the response of the RAW 264.7 cells (1% relative cytotoxicity at 50 μg/mL) showed significantly more sensitivity than that of the CHO cells (60%). CHO cells showed no cytotoxic response to the high fire particles while the RAW 264.7 cells showed a high response (24%). The differential response to the two types of particles is the result of the inherent physical characteristiccs of these two particles. The processing temperatures used to produce these metal oxides yield significantly different particle structures. The high fire particles tend to have a much smoother surface and therefore less surface area than the more porous low fire particles.

Treatment of RAW 264.7 cells with low fire BeO did not result in a dose-related increase in mutant frequency (i.e., mutants per 10^6 clonable cells) over a dose range of 5 to 200 μg/mL, regardless of whether mutant selection was conducted at 5 days or 7 days post-treatment (Table 1). The increase in mutant frequency at 25 μg/mL (day 7 selection) was less than 40 mutants per 10^6 clonable cells above that in the untreated control and was not considered to have any biological significance. It was noted that the mutant frequency in the untreated controls (for both day 5 and day 7 selections) was substantially higher than the spontaneous frequency of between 0 and 10 mutants per 10*6* cells reported by Hitchins *et al.* (1992).

Treatment of RAW 264.7 cells with high fire BeO did not result in a dose-related increase in mutant frequency over a dose range of 100 to 500 μg/mL (mutant selection conducted at 5 days post-treatment, although an increase in mutant frequency (at 100 μg/mL) of 51 mutants per 10^6 clonable cells above that in the untreated control was noted (Table 2). The mutant frequency in the untreated controls was substantially higher than the spontaneous frequency of between 0 and 10 mutants per 10*6* cells reported by Hitchins *et al.* (1992).

The preceding experiments were conducted with treatment of cells in suspension and passage of the cells as suspension cultures until the time of plating for mutant selection. Since the cell generation time was about 18 hours for attached cultures and about 24 hours for suspension cultures, the shorter generation time of attached cultures may decrease the time needed for fixation and expression of mutations. On the other hand, the use of suspension cultures may help to minimize the difficulty of cell removal from flasks with attached cultures. In light of these considerations, an experiment was conducted to compare the effect of passaging cells as

suspension cultures and that of passaging cells as attached cultures following treatment. Low fire and high fire BeO, along with EMS (as positive control) and untreated and solvent controls, were included in this comparative study. As presented in Table 3 (Trial 1) and Table 4 (Trial 2), there was essentially no difference in the results between the use of suspension cultures and attached cultures following treatment in the yield of mutants.

Ethyl Methanesulfonate (EMS). For use as a positive control in the RAW 264.7 mutagenicity assay system, EMS was evaluated in two independent experiments. A dose-related increase in mutant frequency was observed in both experiments (Table 5).

Comparison of RAW 264.7 from Two Different Stock Cultures. To ensure that the stock culture in this laboratory (Stock Culture 2) was functioning in a manner similar to that of cells from another laboratory (Stock Culture 1), the cells from both stock cultures were compared with respect to their response to the cytotoxic and mutagenic effects of EMS and low fire BeO. The mutagen EMS elicited a dose-related increase in toxicity (Table 6) and mutagenicity (Table 7) in the cells from both stock cultures, while low fire BeO was essentially non-toxic (Table 6) and non-mutagenic (Table 7) to cells from both stock cultures.

Trinitrotoluene (TNT)-Contaminated Compost and Uncontaminated Soil Sample. In the initial mutagenicity assay using a 5-hour treatment time, essentially no cytotoxicity and no mutagenic activity were elicited by TNT-contaminated compost, 10% TNT-contaminated compost and uncontaminated soil sample (Table 8, Trial 1). Similar results were obtained with TNT-contaminated compost and uncontaminated soil sample in an independent experiment (Table 8, Trial 2). Using an 18-hour extended treatment time, no mutagenic activity was observed with TNT-contaminated compost at concentrations ranging from 1 to 100 µg/mL and uncontaminated soil sample at concentrations of 1 and 10 µg/mL (Table 9, Trial 1). Upon retesting at higher concentrations (10 to 500 µg/mL), a dose-related increase in mutant frequency was observed with 10% TNT-contaminated compost at concentrations of 100 and 500 µg/mL (Table 9, Trial 2). No mutagenic activity was observed with uncontaminated soil sample in the retest at concentrations of 1 and 10 µg/mL (Table 9, Trial 2). However, the mutant frequency in the untreated controls in this experiment was substantially higher than the spontaneous frequency of between 0 and 10 mutants per 10*6* cells reported by Hitchins *et al.* (1992).

Red Dye Mix. No mutagenic activity was elicited by red dye mix at concentrations ranging from 5 to 100 µg/mL (Table 10).

CONCLUSIONS

The inability to demonstrate mutagenicity with low fire BeO or high fire BeO indicates that the assay procedure still requires further refinement. The mutant frequency in the untreated control in some experiments was substantially higher than the spontaneous frequency of between 0 and 10 mutants per 10*6* cells reported by Hitchins *et al.* (1992). It remains to be determined as to whether the variability in spontaneous mutant frequency is an inherent feature of this cell line, as in the case of Chinese hamster ovary cells in the CHO/HGPRT assay system. No mutagenic activity was demonstrated with red dye mix. A dose-related increase in mutant frequency was observed with 10% TNT-contaminated compost in only one experiment. Since the TNT-contaminated compost was not tested at concentrations higher than 100 µg/mL using the 18-hour

extended treatment regimen, additional experiments are warranted prior to making comments on the potential mutagenicity of soils contaminated with munitions.

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Table 1

Mutagenicity Assay of Low Fire Beryllium Oxide

Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish			Total # Colonies	Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
Day 5 Selection												
Untreated	99	112	122	333	111	59	42	44	49	64	258	93.0
Low Fire Berrilium Oxide												
5 μg/ml	208	118	102	428	143	58	57	51	86	54	306	85.8
10 μg/ml	104	216	139	459	153	50	49	54	55	42	250	65.4
25 μg/ml	106	105	158	369	123	48	65	63	64	65	305	99.2
50 μg/ml	101	126	228	455	152	39	47	47	33	53	219	57.8
100 μg/ml	108	83	106	297	99	47	38	39	49	42	215	86.9
200 μg/ml	106	110	121	337	112	18	22	27	27	20	114	40.6
EMS (positive control)												
0.6 μl/ml	116	182	95	393	131	185	141	184	136	128	774	236.3
DMSO (solvent control for EMS)												
10 μl/ml	122	116	124	362	121	71	48	53	53	57	282	93.5
Day 7 Selection												
Untreated	148	134	168	450	150	45	51	49	44	60	249	66.4
Low Fire Berrilium Oxide												
5 μg/ml	135	174	159	468	156	110	94	85	99	98	486	124.6
10 μg/ml (not plated)												
25 μg/ml	145	145	162	452	151	68	84	81	85	77	395	104.9
50 μg/ml	166	126	139	431	144	66	63	63	65	51	308	85.8
100 μg/ml	150	120	138	408	136	34	36	22	45	28	165	48.5
200 μg/ml	170	167	151	488	163	27	38	43	42	30	180	44.3
EMS (positive control)												
0.6 μl/ml	118	128	136	382	127	202	173	189	206	219	989	310.7
DMSO (solvent control for EMS)												
10 μl/ml	127	150	142	419	140	58	61	69	74	53	315	90.2

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS); EMS = ethyl methanesulfonate.

Table 2

Mutagenicity Assay of High Fire Beryllium Oxide

Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish			Total # Colonies	Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
Day 5 Selection												
Untreated	91	153	133	377	126	50	54	36	42	52	234	74.5
High Fire Berrilium Oxide												
100 µg/ml	68	87	69	224	75	41	55	42	46	51	235	125.9
200 µg/ml	88	94	77	259	86	17	17	19	22	18	93	43.1
300 µg/ml	83	94	69	246	82	38	42	36	34	30	180	87.8
400 µg/ml	72	73	84	229	76	13	7	7	12	11	50	26.2
500 µg/ml	47	69	76	192	64	7	22	11	10	ND	50	39.1
EMS (positive control)												
0.6 µl/ml	52	49	54	155	52	64	52	43	43	66	268	207.5
DMSO (solvent control for EMS)												
10 µl/ml	71	90	78	239	80	46	39	41	39	46	211	105.9

Cloning efficiency = cloning efficiency at time of plating for mutant selection. Untreated Control = Hanks' balanced salt solution (HBSS); EMS = ethyl methanesulfonate; ND = no data.

Table 3

Mutagenicity Assay of High Fire and Low Fire Beryllium Oxide

Using RAW 264.7 Macrophage Cells in 96-Microwell Plates (Trial 1)

Treatment	Viability Assessment Plates			Mutant Selection Plates			
	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Mutants per 10^6 Clonable Cells
Using Cells Passaged as Suspension Cultures							
Untreated	19	96	54.0	184	192	0.0009	15.8
High Fire Berrilium Oxide							
5 μg/ml	18	96	55.8	168	192	0.0027	47.9
50 μg/ml	21	96	50.7	173	192	0.0021	41.1
Low Fire Berrilium Oxide							
5 μg/ml	21	96	50.7	186	192	0.0006	12.5
50 μg/ml	31	96	37.7	174	192	0.0020	52.3
EMS (positive control)							
0.3 μl/ml	20	96	52.3	155	192	0.0043	81.9
1.0 μl/ml	48	96	23.1	129	192	0.0080	344.2
DMSO (solvent control for EMS)							
10 μl/ml	19	96	54.0	172	192	0.0022	40.7
Using Cells Passaged as Attached Cultures							
Untreated	49	192	45.5	373	384	0.0006	12.8
High Fire Berrilium Oxide							
5 μg/ml	28	192	64.2	355	384	0.0016	24.5
50 μg/ml	36	192	55.8	351	384	0.0018	32.2
Low Fire Berrilium Oxide							
5 μg/ml	36	192	55.8	163	192	0.0033	58.7
50 μg/ml	48	192	46.2	372	384	0.0006	13.7
EMS (positive control)							
0.3 μl/ml	23	192	70.7	304	384	0.0047	66.1
1.0 μl/ml	48	192	23.1	149	192	0.0051	219.5
DMSO (solvent control for EMS)							
10 μl/ml	46	192	47.6	354	384	0.0016	34.2

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS); EMS = ethyl methanesulfonate;
- = not tested; ND = no data.

Table 4

Mutagenicity Assay of High Fire and Low Fire Beryllium Oxide

Using RAW 264.7 Macrophage Cells in 96-Microwell Plates (Trial 2)

Treatment	Viability Assessment Plates			Mutant Selection Plates			
	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Mutants per 10^6 Clonable Cells
Using Cells Passaged as Suspension Cultures							
Untreated	4	96	105.9	165	192	0.0030	28.6
High Fire Berrilium Oxide							
5 μg/ml	1	96	152.1	150	192	0.0049	32.5
20 μg/ml	4	96	105.9	142	192	0.0060	57.0
50 μg/ml	1	96	152.1	165	192	0.0030	19.9
Low Fire Berrilium Oxide							
5 μg/ml	4	96	105.9	98	192	0.0135	127.0
20 μg/ml	10	96	75.4	149	191	0.0050	65.9
50 μg/ml	3	96	115.5	144	192	0.0058	49.8
EMS (positive control)							
0.3 μl/ml	12	96	69.3	129	192	0.0080	114.8
1.0 μl/ml	9	96	78.9	4	192	0.0774	981.2
DMSO (solvent control for EMS)							
10 μl/ml	6	96	92.4	156	192	0.0042	44.9
Using Cells Passaged as Attached Cultures							
Untreated	8	96	82.8	188	192	0.0004	5.1
High Fire Berrilium Oxide							
5 μg/ml	15	96	61.9	175	186	0.0012	19.7
20 μg/ml	15	96	61.9	150	188	0.0045	73.0
50 μg/ml	11	96	72.2	132	146	0.0020	27.9
Low Fire Berrilium Oxide							
5 μg/ml	11	96	72.2	189	192	0.0003	4.4
20 μg/ml	11	96	72.2	168	173	0.0006	8.1
50 μg/ml	24	96	46.2	155	161	0.0008	16.4
EMS (positive control)							
0.3 μl/ml	12	96	69.3	147	174	0.0034	48.7
DMSO (solvent control for EMS)							
10 μl/ml	9	95	78.6	165	192	0.0030	38.6

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS); EMS = ethyl methanesulfonate;
- = not tested; ND = no data.

Table 5

Mutagenicity Assay of Ethyl Methanesulfonate (EMS)

Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish		Total # Colonies	Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
Trial 1: Day 7 Selection											
Untreated	134	99	98	331	110	16	12	17	10	16	71	64.4
EMS											
0.2 µl/ml	112	ND	100	212	71	10	10	8	12	6	46	65.1
0.4 µl/ml	102	95	112	309	103	9	24	17	6	13	69	67.0
0.6 µl/ml	71	72	81	224	75	37	37	55	47	47	223	298.7
0.8 µl/ml	70	101	72	243	81	16	11	34	26	21	108	133.3
1.0 µl/ml	91	81	78	250	83	13	11	17	9	16	66	79.2
DMSO (solvent control) 10 µl/ml	100	126	98	324	108	6	5	4	0	8	23	21.3
Trial 6: Day 6 Selection											
Untreated	81	71	96	248	83	16	5	6	7	7	41	49.6
EMS											
0.2 µl/ml	71	92	69	232	77	7	4	5	12	5	33	42.7
0.4 µl/ml	50	70	52	172	57	11	11	6	11	13	52	90.7
0.6 µl/ml	72	73	63	208	69	19	20	16	28	28	111	160.1
0.8 µl/ml	58	71	71	200	67	15	18	14	17	20	84	126.0
1.0 µl/ml	60	79	60	199	66	25	22	20	22	22	111	167.3
DMSO (solvent control) 10 µl/ml	58	64	50	172	57	6	5	5	8	9	33	57.6

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS).

Table 6

Cytotoxicity of Ethyl Methanesulfonate (EMS) and Low Fire Berrilium Oxide in RAW 264.7 Macrophage Cells from Two Different Stock Cultures

	Empty Wells Counted per Dish		Cloning Efficiency (%)	Relative Cloning Efficiency (%)
Stock Culture 1				
Untreated	37	30	35.1	122
EMS				
0.3 µl/ml	35	20	41.7	145
0.6 µl/ml	29	35	36.6	127
1.0 µl/ml	66	53	16.0	55
Low Fire Berillium Oxide				
5 µg/ml	25	33	39.0	139
20 µg/ml	30	30	38.8	135
50 µg/ml	32	34	35.6	124
DMSO (solvent control) 10 µl/ml	41	40	28.8	100
Stock Culture 2				
Untreated	31	33	36.6	96
EMS				
0.3 µl/ml	33	26	39.3	103
0.6 µl/ml	43	47	25.3	66
1.0 µl/ml	78	75	7.6	20
Low Fire Berillium Oxide				
5 µg/ml	38	34	32.7	86
20 µg/ml	25	20	48.4	127
50 µg/ml	28	29	40.5	106
DMSO (solvent control) 10 µl/ml	36	25	38.2	100

Cloning efficiency based on total of 192 wells counted.
Untreated Control = Hanks' balanced salt solution (HBSS).

Table 7

Mutagenicity Assay of Ethyl Methanesulfonate (EMS) and Low Fire Berrilium Oxide in RAW 264.7 Macrophage Cells from Two Different Stock Cultures

Treatment	Viability Assessment Plates			Mutant Selection Plates			
	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Empty Wells Counted	Total # Wells Counted	Cloning Efficiency (%)	Mutants per 10^6 Clonable Cells
Stock Culture 1							
Untreated	41	192	51.5	166	192	0.0029	56.6
EMS							
0.3 µl/ml	30	192	61.9	186	192	0.0006	10.3
0.6 µl/ml	26	192	66.7	165	192	0.0030	45.5
1.0 µl/ml	60	132	38.8	120	176	0.0077	197.6
Low Fire Berillium Oxide							
5 µg/ml	34	158	57.7	174	192	0.0020	34.1
20 µg/ml	45	147	48.4	192	192	0.0000	0
50 µg/ml	85	192	27.2	168	192	0.0027	98.3
DMSO (solvent control) 10 µl/ml	58	192	39.9	185	192	0.0007	18.6
Stock Culture 2							
Untreated	30	192	61.9	161	192	0.0035	56.9
EMS							
0.3 µl/ml	98	192	22.4	164	168	0.0005	21.5
0.6 µl/ml	58	192	39.9	166	192	0.0029	72.9
1.0 µl/ml	18	192	78.9	50	192	0.0269	341.0
Low Fire Berillium Oxide							
5 µg/ml	30	192	61.9	182	192	0.0011	17.3
20 µg/ml	35	192	56.7	169	192	0.0026	45.0
50 µg/ml	41	192	51.5	173	192	0.0021	40.5
DMSO (solvent control) 10 µl/ml	36	192	55.8	163	184	0.0024	43.4

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS).

Table 8

Mutagenicity Assay of Trinitrotoluene (TNT)-Contaminated Compost and Uncontaminated Soil Sample Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish			Total # Colonies	Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
5-Hour Treatment (Trial 1)												
Untreated	137	136	179	452	151	16	11	18	11	12	68	18.1
TNT-Contaminated Compost												
10 µg/ml	157	93	207	457	152	19	20	6	17	ND	62	20.4
50 µg/ml	169	151	153	483	161	15	10	7	10	13	55	13.7
100 µg/ml	146	148	ND	294	147	14	21	17	14	18	84	22.9
500 µg/ml	98	83	107	288	96	5	15	7	19	9	55	22.9
10% TNT-Contaminated Compost												
10 µg/ml	151	157	131	439	146	5	10	11	8	10	44	12.0
Uncontaminated Soil Sample												
10 µg/ml	141	111	162	414	138	5	7	7	4	7	30	8.7
50 µg/ml	82	110	102	294	98	32	29	33	26	22	142	58.0
100 µg/ml	105	105	106	316	105	7	10	10	16	ND	43	20.4
EMS (positive control)												
0.6 µl/ml	113	131	121	365	122	78	59	94	63	70	364	119.7
DMSO (solvent control)												
10 µl/ml	188	195	200	583	194	15	28	22	28	38	131	27.0
5-Hour Treatment (Trial 2)												
TNT-Contaminated Compost												
10 µg/ml	60	63	83	206	69	3	6	2	1	2	14	8.2
50 µg/ml	39	84	98	221	74	10	7	9	7	4	37	20.1
100 µg/ml	101	104	113	318	106	8	6	6	5	10	35	13.2
500 µg/ml	71	81	97	249	83	2	7	5	9	3	26	12.5
Uncontaminated Soil Sample												
10 µg/ml	83	70	64	217	72	15	17	20	12	18	82	45.3
EMS (positive control)												
0.6 µl/ml	40	57	39	136	45	22	28	35	23	20	128	112.9
DMSO (solvent control)												
10 µl/ml	82	94	82	258	86	4	2	1	1	3	11	5.1

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS); ND = no data.

Table 9

Mutagenicity Assay of Trinitrotoluene (TNT)-Contaminated Compost and Uncontaminated Soil Sample Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish			Total # Colonies	Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
18-Hour Treatment (Trial 1)												
Untreated	72	74	97	243	81	4	7	2	5	4	22	10.9
TNT-Contaminated Compost												
1 µg/ml	81	73	88	242	81	1	7	1	1	4	14	6.9
10 µg/ml	80	95	77	252	84	7	8	14	12	8	49	23.3
50 µg/ml	66	70	62	198	66	5	3	2	4	2	16	9.7
100 µg/ml	53	53	42	148	49	3	3	4	6	7	23	18.6
Uncontaminated Soil Sample												
1 µg/ml	69	86	66	221	74	6	7	5	7	10	35	19.0
10 µg/ml	80	77	83	240	80	9	12	12	7	6	46	23.0
EMS (positive control)												
0.1 µl/ml	82	98	93	273	91	31	34	27	21	20	133	58.5
0.3 µl/ml	36	46	52	134	45	121	108	111	124	118	582	521.2
DMSO (solvent control)												
10 µl/ml	77	69	74	220	73	9	4	3	12	7	35	19.1
18-Hour Treatment (Trial 2)												
Untreated	118	129	122	369	123	36	47	36	52	36	207	67.3
10% TNT-Contaminated Compost												
100 µg/ml	106	117	111	334	111	35	70	58	50	62	275	98.8
500 µg/ml	87	93	129	309	103	111	93	131	128	125	592	229.9
Uncontaminated Soil Sample												
1 µg/ml	69	86	66	221	74	6	7	5	7	10	35	19.0
10 µg/ml	80	77	83	240	80	9	12	12	7	6	46	23.0
EMS (positive control)												
0.4 µl/ml	22	49	41	112	37	17	34	20	31	ND	102	136.6
DMSO (solvent control)												
10 µl/ml	134	145	134	413	138	37	46	57	62	69	271	78.7

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS); ND = no data.

Table 10

Mutagenicity Assay of Red Dye Mix Using RAW 264.7 Macrophage Cells

Treatment	Colonies per Dish		Total # Colonies		Cloning Efficiency (%)	Mutant Colonies per Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants per 10^6 Clonable Cells
Untreated	121	130	101	352	117	13	16	14	19	9	71	24.2
Red Dye Mix												
5 μg/ml	141	140	145	426	142	10	15	6	8	5	44	12.4
10 μg/ml	106	99	138	343	114	8	5	5	9	10	37	12.9
50 μg/ml	161	127	132	420	140	5	8	4	7	8	32	9.1
100 μg/ml	180	220	239	639	213	10	6	8	14	13	51	9.6
EMS (positive control)												
0.6 μl/ml	172	175	131	478	159	51	54	55	56	64	280	70.3
DMSO (solvent control)												
10 μl/ml	165	185	193	543	181	17	12	15	10	13	67	14.8

Cloning efficiency = cloning efficiency at time of plating for mutant selection.
Untreated Control = Hanks' balanced salt solution (HBSS).